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Expression and Localization of Prohormone Convertase 1/3 (SPC3) in Porcine Ovary

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ABSTRACT Tissue distribution and cellular localization of PC1/3 mRNA in porcine tissues were examined by ribonuclease protection assay and in situ hybridization. PC1/3 mRNA was detected mainly in the corpus luteum of pregnant sow and brain. Within the ovary, PC1/3 and relaxin transcripts colocalized within large luteal cells. Levels of PC1/3 transcripts in corpora lutea increased as gestation advanced, parallel with an observed increase in relaxin transcripts. A role for PC1/3 in proprotein processing in the ovary is discussed. *Mol. Reprod. Dev. 57:361–365, 2000.*

Key Words: prohormone convertase; PC1/PC3; ovary; gene expression; localization

INTRODUCTION

Prohormone convertase 1/3 (SPC3) is a member of the subtilisin-like proprotein convertases which are involved in the posttranslational processing of many prohormones and precursors of other biologically active proteins (Steiner 1998; Seidah and Chretien 1999). So far seven members of these proprotein convertases have been identified; they are furin/PACE (SPC1), PC2 (SPC2), PC1/3 (SPC3), PACE4 (SPC4), PC4 (SPC5), PC5/6 (SPC6), and PC7/8 or LPC (SPC7). Among them, furin, PACE4, and PC7/8 are ubiquitously expressed (Hatsuzawa et al., 1990; Tsuji et al., 1994; Bruzzaniti et al., 1996), whereas expression of PC1/3 and PC2 is restricted to neuroendocrine tissues (Seidah et al., 1990; Smeekens et al., 1991). PC4 is expressed primarily in testis (Nakayama et al., 1992). PC5/6 is expressed at highest level in gastrointestinal tissues, but is also expressed at lower levels in other tissues (Lusson et al., 1993; Nakagawa et al., 1993). Recently, we have cloned PC1/3 from a porcine ovary cDNA library (Dai et al., 1995). In the current study, we investigated the tissue distribution and cellular localization of PC1/3 transcripts in porcine tissues, and found that PC1/3 was expressed mainly in the corpus luteum of pregnant sow and brain, and that its levels in the corpus luteum increased progressively as gestation advanced.

MATERIALS AND METHODS

Materials

PC327 and PC323 are partial clones of porcine PC1/3 (Dai et al., 1995). PC421 is a subclone of the 200 bp EcoRI/StuI fragment of PC323, containing the coding sequence of residues 272–338 of PC1/3. PC 422 is a subclone of the 320 bp EcoRI/ApaI fragment of PC327, containing the coding sequence of residues 164–269 of PC1/3. PR423 is a subclone of the 230 bp EcoRI/BglII fragment of porcine preprorelaxin cDNA, pPR308-6 (Reddy et al., 1992). It contains 5′-untranslated sequence and coding sequences of prepeptide and 20 residues of the relaxin B-chain. RP440 is a subclone of the 440 bp EcoRI/XhoI fragment of porcine ribosomal protein S17 cDNA, RP302, containing the coding sequence of 146 residues at the N-terminus (Kwok, unpublished result).

Ribonuclease Protection Assay

The ^{32}P-labeled, antisense riboprobes of PC422, PR423, and RP440 were produced from the corresponding plasmids using the MAXIscript In Vitro Transcription Kit (Ambion Inc., Austin, TX), and were purified on a 5% polyacrylamide gel containing 8 M urea according to the protocol provided by the manufacturer. RP 440 was used as an internal control for uniformity of RNA sampling among tissues and days of pregnancy, and PR423 was included as a positive control. Total RNA was extracted from porcine stomach, heart, kidney, lung, brain, intestine, liver, seminal vesicles, prostate, epididymis, and ovary according to Chomczynski and Sacchi (1987). Ribonuclease protection assays were performed according to the protocol provided with the RPA II Ribonuclease Protection Assay Kit (Ambion Inc., Austin, TX). Briefly, 10 or 50 μg of total RNA were

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coprecipitated with a mixture of the three antisense riboprobes (10^5 cpm each), heat-denatured in 20 μl of hybridization buffer and allowed to hybridize at 45°C overnight. Unhybridized riboprobes were digested with a mixture of RNase A/T_1 and the protected fragments were separated on a 5% polyacrylamide gel containing 8 M urea, and visualized by autoradiography.

Northern Blot Analysis

Antisense ^{32}P-labeled riboprobe was generated from plasmid PC327 using a Riboprobe System-T7 kit (Promega, Madison, WI). Total RNA was extracted from porcine ovaries according to Chomczynski and Sacchi (1987) and 10 μg was separated using a 1.5% agarose gel. RNA was capillary transferred to nylon membrane (Hybond-N, Amersham, Piscataway, NJ) and then stabilized by UV-crosslinking and heating at 80°C. Immobilized RNA was incubated overnight at 60°C in hybridization buffer (5× SSC, 5× Denhardt's solution, 0.5% SDS, 50% formamide) containing the ^{32}P-labeled riboprobe, and unhybridized probe was removed by washing the blot with 0.1× SSC (65°C). The hybridized bands were visualized by autoradiography.

In Situ Hybridization Histochemistry

One cubic-centimeter^3 pieces of ovary were frozen by immersion in isopentane cooled (−40°C) with dry ice, sectioned (12 μm), and mounted on gelatin/chromium potassium sulfate coated slides. Sections were fixed in 4% paraformaldehyde, acetylated and hybridized at 50°C for 4 hr in hybridization buffer (4× SSC, 50% formamide, 10% dextran sulfate, 1× Denhardt's solution) containing 1–3 ng of ^{35}S-labeled antisense or sense riboprobes. Porcine PC1/3 (PC421) and relaxin (PR423) specific probes were generated using a Riboprobe System-T7 kit (Promega, Madison, WI). After hybridization, the unbound probe was removed by washing (4 × 15 min) with 2× SSC/50% formamide at 40°C and finally with 1× SSC at RT.

Air-dried sections were exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY). Hybridization signals were analyzed after capturing (Data Translation Frame Graber) film images from a Northern Light Box (Imaging Research, Ontario, Canada) with a Sierra Scientific CCD camera, Image software (Wayne Rasband, NIMH) and a Macintosh Iici computer. After correcting for nonuniform illumination and subtracting film background by density slicing, the C^{14} standards (American Radiolabeled Chemical, Inc., St. Louis, MO) were measured and calibrated to ^{35}S equivalences. A standard curve was generated by plotting transmittance values against known dpm/mg, using a 3rd degree polynomial equation as previously described (Young et al., 1986). This standard curve was then used to calibrate each film. Quantitative differences were expressed as mean density of tissue in dpm/mg. Measurements were taken using an oval field sized to include the entire area of 4 sections/2 or 3 blocks/2 animals. Values were analyzed by ANOVA (NCSS, Kaysville, Utah) for effect of day of gestation.

Following film autoradiography, sections were coated with Kodak NTB-2 nuclear track emulsion and exposed for 3 (relaxin) or 14 (PC1/3) days. Developed slides were counterstained with hematoxylin.

RESULTS

The expression of PC1/3 in various tissues was examined by ribonuclease protection assay (RPA) using an antisense PC422 riboprobe. Of the eleven tissues examined, a protected fragment of PC422 probe was detected only in brain and ovarian RNA samples (data not shown), suggesting that PC1/3 was primarily expressed in these tissues. In the ovary, although PC1/3 was not detected on day 12 of gestation, significant quantities of transcript were present at day 61 and levels increased by late gestation (Fig. 1, top panel, lanes 1–3). Relaxin was expressed at a much higher level than PC1/3, however the temporal pattern of expression was similar to that for PC1/3 (Fig. 1, bottom panel, lanes 1–3).

A single band of approximately 4.4 kb corresponding to PC1/3 was identified in total RNA from pregnant sow ovary (Fig. 2). In situ hybridization histochemistry (ISHH) demonstrated that PC1/3 mRNA was detected at its highest levels in the corpus luteum, although levels were not as high as that for relaxin (Fig. 3A–D). Cells that expressed PC1/3 contained large ovoid to round nuclei surrounded by extensive cytoplasm, which is typical of large luteal cells in the pig (Fig. 3E,F). Similar observations were made for cells that expressed relaxin (data not shown). Consistent with RPA, ISHH demonstrated increasing levels of PC1/3 mRNA between mid and late gestation, as evidenced by an apparent increase in the numbers of emulsion grains/cell (Fig. 3G,H). Also, quantitative analysis of PC1/3 mRNA in luteal sections by ISHH and film autoradiography indicated that the level (mean density) of PC1/3 mRNA increased ($P \leq 0.05$) approximately 35% (average of two experiments) from day 45 to day 90. In addition to the corpus luteum, weak signal for PC1/3 was also found in the stroma and thecal tissue of the ovary (Fig. 3D).

DISCUSSION

The expression of PC1/3 is apparently restricted to neuroendocrine cells (Seidah et al., 1990; Smeekens et al., 1991). Although PC1/3-like transcripts of various sizes have been detected by northern blot analysis in lung, skeletal muscle, and heart (Seidah et al., 1992), the identity of these transcripts as PC1/3 has not been established. Previously described PC1/3 transcripts in the ovary (Marriott et al., 1992) have recently been identified unequivocally as PC1/3 by cloning and sequencing (Dai et al., 1995). In the current study, we further demonstrated that PC1/3 is expressed mainly in the corpus luteum of the ovary and that the levels of PC1/3 transcripts increase as gestation advances. Relaxin mRNA levels also increased as gestation progressed in agreement with a previous report (Bagnell et al., 1990), and PC1/3 and relaxin were colocalized to the large luteal cell. Whether the parallel expression

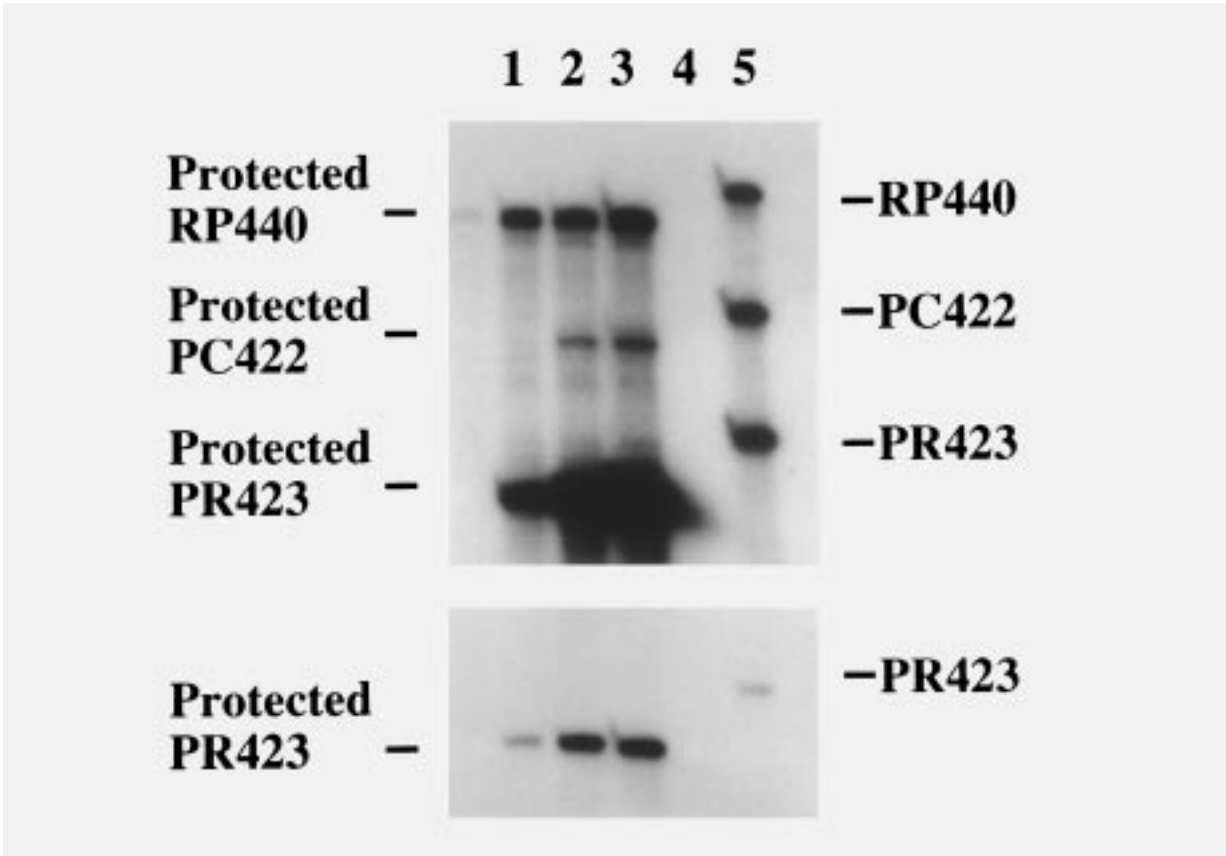

Fig. 1. Ribonuclease protection assay using a mixture of antisense riboprobes of ribosomal protein S17 (RP440; 440 bp), PC1/3 (PC422; 320 bp), and relaxin (PR423; 230 bp). Lanes 1–3 contain RNA from day 12, 61, and 101 pregnant sow ovaries, respectively. Lane 4 contains yeast RNA. Lane 5 contains 1/10 of the undigested riboprobes only. Bottom panel shows the PR423 region of the same gel after a short autoradiographic exposure. Note the parallel increase in expression of PC1/3 and relaxin in porcine ovaries from early to late pregnancy, and the constant levels of ribosomal protein S17.

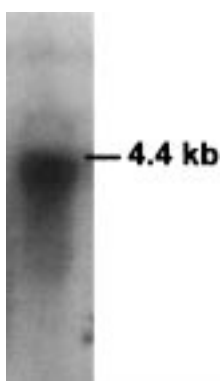

Fig. 2. Northern blot analysis of total RNA from ovary at day 45 of gestation using a PC1/3 riboprobe. Note the predominant band at approximately 4.4 kb.

and colocalization of relaxin and PC1/3 transcripts has any functional significance remains to be determined.

Marriott et al. (1992) reported that when cDNAs encoding human preprorelaxin and mouse PC1/3 were coexpressed in human kidney 293 cells, correct processing of human prorelaxin was observed. PC1/3 alone seems to be able to process human prorelaxin. However, participation of other proprotein convertases (such as PC2) has not been ruled out. Our preliminary study using RT-PCR and human ovary cDNA showed that ovary indeed expressed furin, PC2, PACE4, PC5/6, and PC7/8 besides PC1/3 (data not shown). Since all these proprotein convertases cleave at paired basic residues, processing by proprotein convertases may not be fully applicable to porcine and other prorelaxins. With the exception of human and monkey prorelaxins (Hudson et al., 1984; Crawford et al., 1989; Evans et al., 1994), paired basic residues required for recognition and cleavage by PC1/3 and other proprotein convertases are not present at the B-chain/C-peptide junction in other prorelaxins. Hence, processing by PC1/3 and other proprotein convertases at this junction is unlikely and the participation of another processing endoproteinase must be invoked. A possible candidate is SKI-1, which is able to cleave a proprotein at a recognition sequence such as RGLT↓SL or RSVL↓SF (Seidah et al., 1999). Porcine prorelaxin has a sequence of RTAL↓SL at the B-chain/C-peptide junction (Haley et al., 1982). It will be interesting to see if SKI-1 is expressed in the

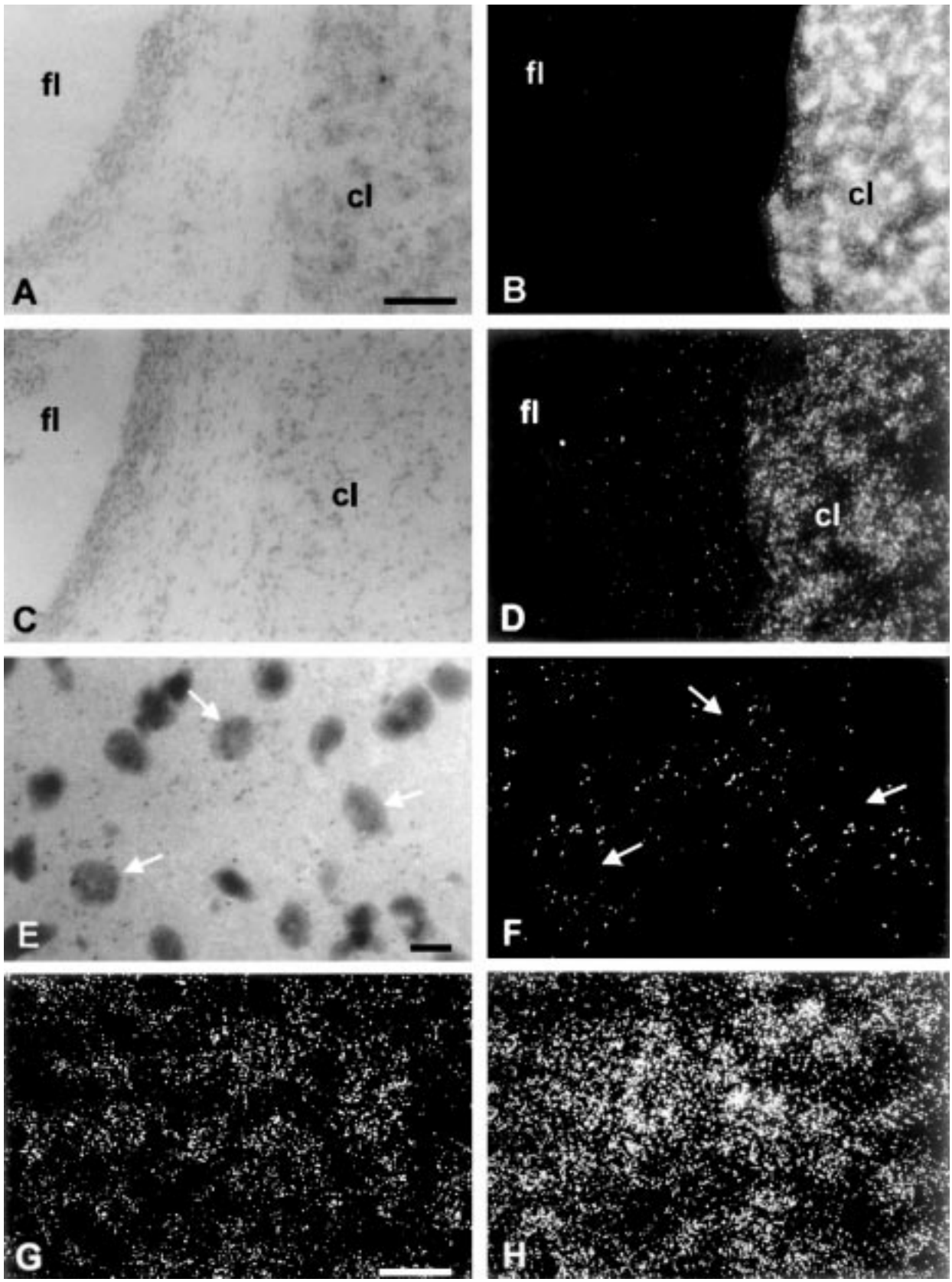

Fig. 3. Cellular localization of PC1/3 and relaxin mRNAs within the pregnant sow ovary. (**A**) Brightfield and (**B**) darkfield micrographs of a section of day 90 pregnant ovary tissue hybridized with the porcine relaxin probe (PR423). Fl, follicle lumen; cl, luteal cells. Bar = 100 μm. (**C**) Brightfield and (**D**) darkfield images of a section adjacent to that in (A) and (B) hybridized with the porcine PC1/3 probe (PC421). (**E**) Brightfield and (**F**) darkfield micrographs of day 45 pregnant sow ovary hybridized with the PC1/3 probe (PC421). Arrows indicate location of luteal cell nuclei surrounded by hybridization signal. Bar = 10 μm. Darkfield micrographs of PC1/3 labeling in the corpus lutea of day 45 (**G**) and day 90 (**H**) pregnant sow ovaries. Note the increased signal with advancing pregnancy. Bar = 50 μm.

pregnant sow ovary and its expression is parallel to that of PC1/3. On the other hand, several other peptides including oxytocin, arginine vasopressin, relaxin-like factor (RLF) and relaxin/insulin-like factor (RIF) are synthesized as prohormones by luteal cells (Einspanier et al., 1986; Watkins and Choy, 1988; Ivell, 1997; Hsu, 1999). PC1/3 may be involved in posttranslational processing of these prohormones. Recently, Guillou et al. (1994) reported the presence of prooxytocin/neurophysin convertase activity in the ovary but molecular identification of this activity was not evaluated. Whether this enzyme is identical to PC1/3 remains to be determined.

Although two forms of PC1/3 mRNAs (3 and 5 kb) have been reported in various species (Creemers et al., 1992; Seidah et al., 1992; Gorham et al., 1996; Gangnon et al., 1999), only one 4.4 kb band was detected in porcine ovarian RNA in this study. Previously, we have isolated five different cDNAs with heterogeneity at the polyadenylation site, resulting in a size of 4.6–5.0 kb (Dai et al., 1995). The 3 kb mRNA was not detected. Whether this is unique to porcine ovarian PC1/3 mRNA remains to be established.

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